

Chemical defense: Aquatic beetle (*Dineutes hornii*) vs. fish (*Micropterus salmoides*)

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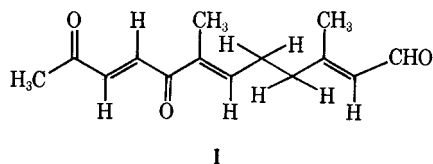
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Captive largemouth bass (*Micropterus salmoides*) reject the gyrinid beetle, *Dineutes hornii*. They also reject edible items (mealworms) treated by topical addition of the norsesquiterpene gyrinidal, the principal component of the defensive secretion of the beetle. The bass' oral tolerance of gyrinidal varies broadly as a function of the gyrinidal dosage and the state of satiation of the fish. When taking a *D. hornii* or a gyrinidal-treated mealworm in the mouth, the fish subjects the item to an intensive oral flushing behavior, seemingly intended to rid the item of gyrinidal. The duration of oral flushing is itself a function of the gyrinidal dosage and the state of satiation of the bass. To counter oral flushing, *D. hornii* emits its secretion as a slow trickle. Duration of emission is slightly longer (1.5 min) than the time (1.3 min) invested by the bass in flushing a *D. hornii* before rejecting the beetle. We postulate that flush resistance may be a general feature of defensive chemical delivery systems in aquatic prey, given that oral flushing may be a common strategy of fish.

Gyrinidae | largemouth bass | gyrinidal | repellent | oral flushing

Insects are primitively terrestrial, but substantial numbers have secondarily adapted to life in fresh water, where they have come face to face with fish. Not surprisingly, some aquatic insects are chemically protected against predation. They possess defensive glands from which they eject noxious fluids when disturbed, fluids containing compounds that have in some cases been characterized. Diving beetles (Dytiscidae) (1), for instance, and belostomatid bugs (Belostomatidae) (2) produce steroids, primarily pregnanes (C21-steroids), shown to have strong defensive potential against fish (1, 3–5).[§]

Also investigated has been the defensive chemistry of the so-called whirligig beetles (Gyrinidae). A familiar sight on ponds and slow streams, these insects are surface swimmers, commonly found by the thousands in dense and conspicuous aggregations (6). Gyrinids have a pair of defensive glands, opening at the tip of the abdomen (7) (Fig. 1). Schildknecht *et al.* (8) and Meinwald *et al.* (9, 10), have independently characterized the principal component of the secretion of two genera of whirligigs, *Dineutes* and *Gyrinus*, to be the highly oxidized norsesquiterpene *gyrinidal* (Scheme 1). Later inves-



tigations revealed the presence of additional compounds in the secretion of some gyrinids, including norsesquiterpenes closely related to gyrinidal (isogyridal, gyrinidone, gyrinidione) (11). Evidence had shown gyrinid beetles to be rejected by fish (12) and gyrinidal and its isoprenoid relatives to be toxic to fish (4, 5), but no investigation had been made of whether these compounds actually repelled fish, as they would certainly need to do to be effective.

We now have investigated the predator–prey interaction of a whirligig beetle, *Dineutes hornii* (henceforth called *Dineutes*) and largemouth black bass, *Micropterus salmoides* (henceforth called *Micropterus* or bass), with the intent of focusing on the function of gyrinidal, the principal isoprenoid produced by this beetle (9). We found that gyrinidal is indeed repellent to the fish, but to a degree that is highly variable, and is a function of the fish's state of satiation. We demonstrate further that the fish, in apparent efforts to rid intended food items of gyrinidal, holds the items in the mouth and subjects them to what we take to be an intensive oral cleansing behavior. The duration of this behavior, which for operational purposes we shall call oral flushing, is also variable, and depends on both the state of satiation of the fish and the quantity of gyrinidal in the food item. Finally, we demonstrate that the beetle, in what appears to be a counteradaptation to the flushing behavior of the fish, doles out its defensive secretion as a slow trickle. Here we present these results, which we feel may have a bearing on the understanding generally of predator–prey interactions in water.

Materials and Methods

Statistics. All values are given as mean + SE.

The Beetles. The *Dineutes* were collected in ponds in the vicinity of Ithaca, Tompkins County, NY. To prevent them from discharging prematurely, they were taken by net and transferred without being touched to a small aquarium for transport to the laboratory. There they were maintained in larger aquaria and used in tests with fish within at most a few days after capture.

The Fish. The bass [body mass = 40 ± 4 g; range = 7–60 g ($n = 17$)] were taken by net in various ponds near Ithaca, including some of the very ponds where the *Dineutes* were collected. They were kept individually in 5-gallon aquaria. The water was continuously aerated (except during feeding sessions) and was changed once a week.

The bass learned quickly to feed on mealworms (larvae of the beetle, *Tenebrio molitor*) that were offered to them with forceps on the water surface in a corner of the aquarium. In a matter of days, they adapted to a diet of about 20 mid-sized mealworms, presented one after the other in a single feeding session every second day. Once adapted to this regimen, they were judged to be ready for experimentation. On days when the experiments were done, the regular feeding sessions of the fish were omitted. No fish was used in more than one experiment. After completion of the study, the fish were returned to their ponds of origin.

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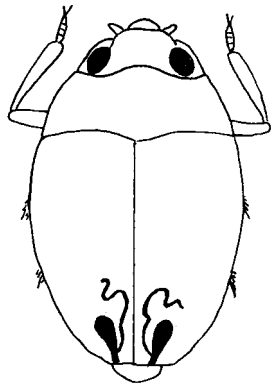


Fig. 1. Diagram of a gyrid beetle showing defensive glands in rear of abdomen. Glands consist of a sac-like reservoir in which secretion is stored and an attached strand of glandular tissue.

Oral Flushing. We witnessed the oral flushing behavior first when we fed *Dineutes* to the bass and subsequently quite regularly when we presented secretion-treated or gyridal-treated mealworms to the fish. We initially took the behavior to be a gagging syndrome, indicative perhaps of impending emesis, but because it did not lead to vomiting, we discarded that interpretation and came to believe instead that it was a cleansing action. The behavior had been noted by others, including in *M. salmoides*, and had variously been referred to as buccal flushing or spitting behavior (1, 12, 13).

The behavior is of abrupt onset and is stereotyped (Fig. 2 A–E). As soon as the fish takes the noxious item in the mouth, it begins to open and close its mouth in a slow rhythmic action, undertaken in more or less regular alternation with an opening and closing of the opercular flaps (the gill covers). While undertaking the behavior, the fish retains the food item in the buccal cavity (without moving it to the pharynx) but usually not continuously. Typically it spits the item out one or more times, taking it into the mouth again on each occasion (usually in a matter of seconds) and then proceeding with the flushing action. The behavior ends with the item being either swallowed (its disappearance from the buccal cavity is usually visually apparent) or spat out in a final rejection (failure to reingest the item within 1 minute was the criterion for rejection).

We quantified the flushing behavior by timing its duration with individual food items. The periods when an item was temporarily spat out during the course of the behavior were not tallied. Flushing times ranged from seconds to over several minutes and could therefore be timed accurately with a hand-held stopwatch.

Satiation. All experimental feeding sessions were continued—that is, food items were presented one after the other—until the fish were satiated. Point of satiation was judged to have been reached when a fish failed for the first time in the sequence of presentations to respond within 1 minute to an untreated (control) mealworm. Sessions were terminated when fish were satiated.

Percent Satiation. This parameter provided a measure (in experiment 3) of the state of repletion of a fish at any point along a feeding session. It was calculated by dividing the number of mealworms (both treated and untreated) eaten up to that point by the total number eventually consumed to achieve satiation and multiplying by 100.

Acceptability of Beetles (Experiment 1). Four bass were fed to satiation on five separate days each. Each day they were given a mix of live *Dineutes* and mealworms (controls), in a ratio of 1:3. The items were presented one at a time, in such sequence that *Dineutes* recurred once at random among mealworms in each consecutive group of three items. A total of 197 mealworms and 96 *Dineutes* were offered to the four fish. Fate of the individual food items was scored as follows: eaten (if it was ingested without preliminary flushing); eaten after flushing (if it was swallowed after first being held in the mouth and flushed); rejected (if it was taken in the mouth and spat out, either right away or after flushing); and ignored (if it was left untouched for at least 1 minute.)

Duration of Oral Flushing with Beetles and with Secretion-Treated Mealworms (Experiment 2). Seven fish were each offered a combination of *Dineutes* and control mealworms in a ratio of 1:3 as in experiment 1, for one or more daily feeding sessions per fish. On another set of days, the same fish were fed in accord with the same regimen, but with secretion-treated mealworms in lieu of the *Dineutes*. The purpose was to time the duration of oral flushing elicited in the fish by exposure to either *Dineutes* or its secretion. The assumption was that the secretion would in itself induce flushing.

Treatment of the mealworms was effected by excising the two defensive glands from individual *Dineutes* killed by freezing and smearing both glands topically on the mealworms just before these were offered to the fish. Excision of the glands was effected simply by pulling the abdominal tip from the beetles; this exposed the glands without tearing them, making it possible to transfer them intact to the mealworm surface.

A total of 12 *Dineutes* and 29 treated mealworms were taken into the mouth by the fish and orally flushed.

Deterency of Gyridal (Experiment 3). To check into the deterrent effectiveness of synthetic gyridal itself, six bass were individually tested in seven feeding sessions each, in which they were offered a series of mealworms, of which the experimentals bore a topical coating of gyridal. In each session, experimental and control mealworms were presented in a ratio of 1:4, in randomized sequence, until the fish were satiated. Gyridal was applied to the experimental mealworms with a micropipette in dichloromethane solution (0.8 μ l). The controls were treated by topical addition of the equivalent volume of dichloromethane only. Gyridal was tested at 7 topical dosages: 0.5, 1.0, 5.0, 15, 35, 100, and 500 μ g. One dosage was tested per feeding session. The sessions testing the two highest dosages (100 and 500 μ g) differed from the other sessions in that, because of shortage of synthetic gyridal, less than the full number of treated mealworms were interspersed in the feeding sequence.

This experiment permitted determination of the relationship of (i) gyridal dosage to acceptability of food item; (ii) gyridal dosage to duration of oral flushing; and (iii) oral tolerance of gyridal to the state of satiation of the fish.

Duration (Fade-Out Time) of the Beetle's Secretory Discharge. Under a microscope, the *Dineutes* secretion is seen to consist of a fine emulsion (Fig. 2K), accounting very probably for its white coloration and yogurt-like consistency. We presume gyridal makes up the inner phase of the emulsion. Casual observation had shown the secretion to be released, not as a burst, but gradually over time. To obtain a measure of the duration of the emission, individual *Dineutes* were held in forceps and stimulated by pinching, while being held submerged in a stream of water (flow rate = 16 cm/sec) and kept under observation with a stereomicroscope.

The forceps had rubber-coated tips to ensure that the smooth-surfaced beetles would not slip from the grasp. Beetles were

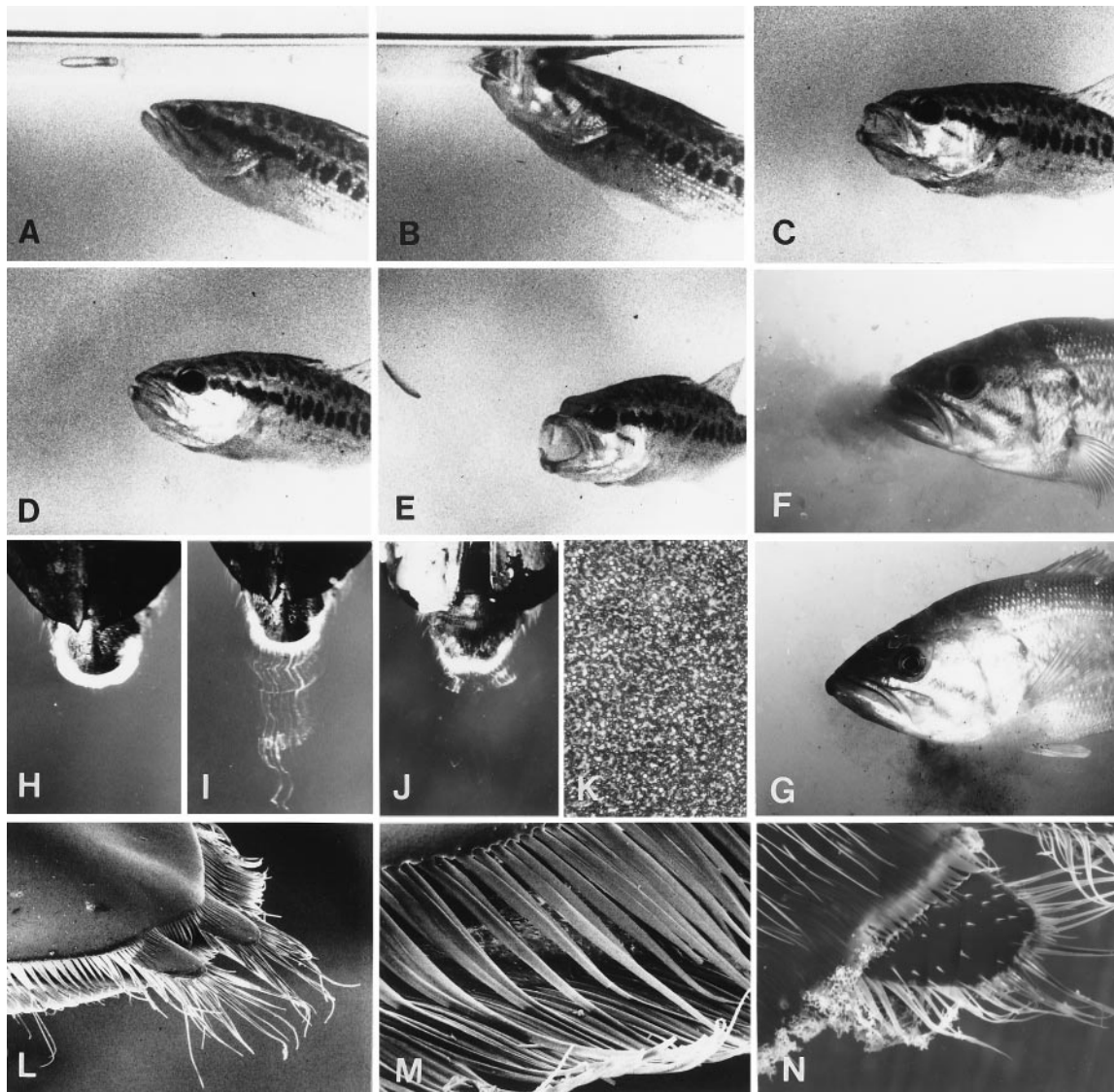


Fig. 2. (A–E) Frames from motion picture film, showing bass taking a gyrinidal-treated mealworm (A, B), then subjecting it to oral flushing (C, D), and finally spitting it out (E). (F, G) Bass subjecting a charcoal-tainted mealworm to oral flushing; the direction of water flow, alternatively out through the mouth (F) and through the operculum (G), is rendered visible by the charcoal marker. F and G correspond to actions depicted in C and D, respectively. (H–J) Rear of *Dineutes* beetles in the process of ejecting secretion. The white fluid collects initially on the margin of the pygidium (H), then drifts backward with the water current (I), while being bolstered occasionally by paired “puffs” of secretion (J). (K) Magnified view of *Dineutes* secretion; gyrinidal is presumed to be a part of the finely dispersed inner phase of the emulsion. (L) Rear of *Dineutes* beetle showing the interdigitating hairs that form the pygidial “sieve” in which the secretion collects when discharged; projecting through the hairs are the two palettes that presumably help force secretion through the sieve, giving rise to “puffs” (J). (M) Detail of preceding. (N) Comparable to L, but of a beetle that was killed by freezing while emitting secretion, and was then freeze dried. Remnants of secretion are seen clinging to the hairs of the pygidial sieve and to one of the palettes. (A, $\times 0.3$; H, $\times 8$; K, $\times 340$; L, $\times 40$.)

either given a single pinch (5 males, 6 females), or were pinched on an ongoing basis at the rate of about twice per second while being held (10 males, 10 females). In either case, observation was continuous, and the beetles were held in the forceps for as long as secretion could be seen drifting from their rear. The duration of this emission (the signal fade-out time) was timed with a foot-operated stopwatch from the moment (usually within seconds after the pinch or the onset of the pinch train) that secretory output became apparent.

Results and Conclusions

Acceptability of Beetles (Experiment 1). As is seen from the results (Fig. 3), only 3 of the 96 *Dineutes* offered were eaten. The three beetles were consumed by one fish and were each subjected to oral flushing before being swallowed. Another 17 beetles were

rejected, either outright or after flushing, and the remaining 76 were ignored. The rejected beetles all survived uninjured. The control mealworms were all swallowed outright without first being flushed.

Duration of Oral Flushing with Beetles and with Secretion-Treated Mealworms (Experiment 2). Of the 12 beetles that were seized by the fish, all were rejected, and all were orally flushed before being released. The flushing time was 78 ± 30 sec and ranged widely from 4 sec (3 beetles) to 330 sec.

The 29 treated mealworms were all eaten by the fish but were in each case first flushed. Flushing time was 23 ± 2 sec and showed relatively little variation (range: 10–50 sec).

It is clear from these results that the secretion itself of *Dineutes*, as evidenced by its effect on addition to the meal-

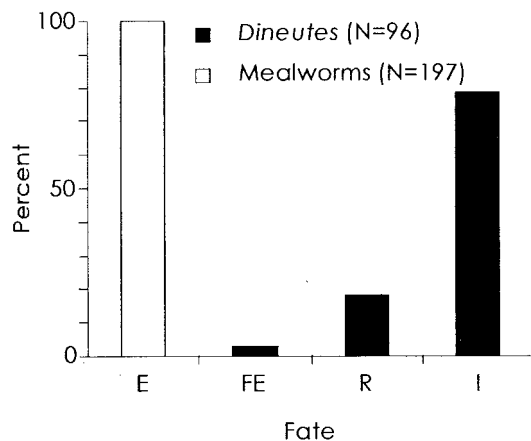


Fig. 3. Fate of live *Dineutes* and of untreated mealworms offered to bass (data from six fish are lumped). E, eaten outright; FE, eaten after flushing; R, rejected outright or after flushing; I, ignored.

worms, can induce oral flushing. It is equally clear, given the fate of these mealworms, that oral flushing can result in food items being rendered eventually acceptable. The beetles, on the other hand, were rejected despite flushing, indicating that the cleansing procedure, no matter how long, is not always effective. It is also worth noting, given that the beetles were rejected live, that they were not subjected to any injurious processing, either in the mouth or pharynx of the fish, during flushing.

Visualization of Oral Flushing. Several fish were fed individual mealworms that were coated beforehand with a thick pasty mixture of Cremora (Borden), water, and powdered animal charcoal. Just before presentation to the fish, a small quantity of gyrinidal was added to the mealworm coating to induce oral flushing. The coating was expected to be washed off during flushing and to reveal, by its black marker, how water flows back and forth through mouth and gill cavities during the procedure. As is evident from Fig. 2 *F* and *G*, the technique served its purpose.

Deterrence of Synthetic Gyrinidal (Experiment 3a). Taken together, the data permitted calculation of the dose dependency of the deterrence of gyrinidal. For each gyrinidal dosage tested (but excluding the tests with 100 and 500 μg), we lumped the data from the six fish and calculated the percent of total treated mealworms that were rejected. The results (Fig. 4) show that even at a dosage of 35 μg gyrinidal, amounting to a fraction of the 100 μg stored by individual *Dineutes*, nearly half the mealworms were rejected.

We excluded the data obtained with the two high dosages not only because of insufficiency of data points but also because of unusual behavior of two of the fish that had ingested treated mealworms bearing these dosages. In each case, these fish vomited the entire quantity of mealworms they had so far ingested in the feeding session, an effect that could well have been a consequence of the known toxicity (4, 5) of gyrinidal.

Relationship of Duration of Oral Flushing to Dosage of Gyrinidal (Experiment 3b). For each dosage, we lumped the data from the six fish and calculated the mean duration of oral flushing to which the treated and untreated mealworms were subjected. It is clear from the results (Fig. 5) that the higher the dosage, the longer the time the fish invests in flushing. It is clear also that flushing times, for each dosage, were highly variable.

The fish also spent short periods of time flushing when given untreated control mealworms. These times were longest in the

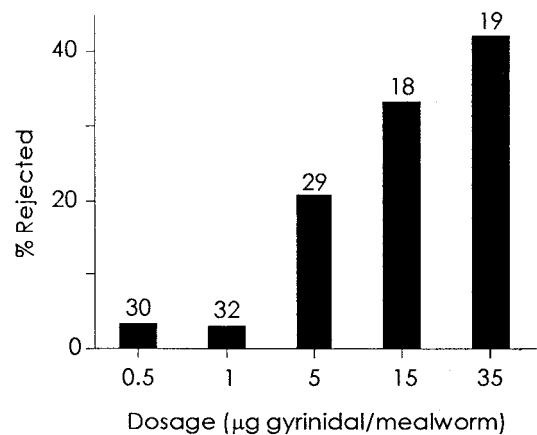


Fig. 4. Percent rejection of mealworms by bass, plotted as a function of the dosage of synthetic gyrinidal applied topically to the mealworms. Data from six bass are lumped. Numbers above columns give sample sizes.

test sessions with the highest gyrinidal dosages, indicating perhaps that in these sessions the fish never entirely rinsed their mouths of gyrinidal and that, when flushing controls, they were responding to an aftertaste of the chemical. Alternatively, because controls were visually indistinguishable from experimentals in these sessions, it could be that the fish, by generalizing from the noxious experience with the treated mealworms, were coming to “suspect” all mealworms to be in need of cleansing, with the result that they then flushed, or at least briefly flushed, every mealworm.

Relationship of Oral Tolerance of Gyrinidal to State of Satiation of the Fish (Experiment 3c). The data expressing this relationship are plotted individually for the six fish in Fig. 6. For each fish, a separate calculation was made for every gyrinidal dosage tested of the state of satiation above which no further treated items were accepted. That state of satiation was assumed to lie midway between the states of satiation that prevailed when a treated item was last accepted and when it was first rejected. These two points of satiation are linked by the vertical lines in the plots, and the calculated midpoints are given by the transverse lines. In cases

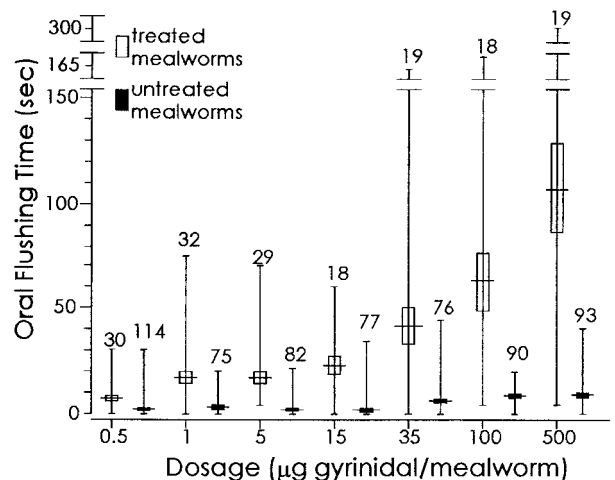


Fig. 5. Length of time bass subjected mealworms to oral flushing, plotted as a function of dosage of synthetic gyrinidal applied topically to the mealworms. Data are also given for times spent flushing with untreated control mealworms. Data from six bass are lumped. Values are given as mean + SE, plus range. Numbers give sample sizes.

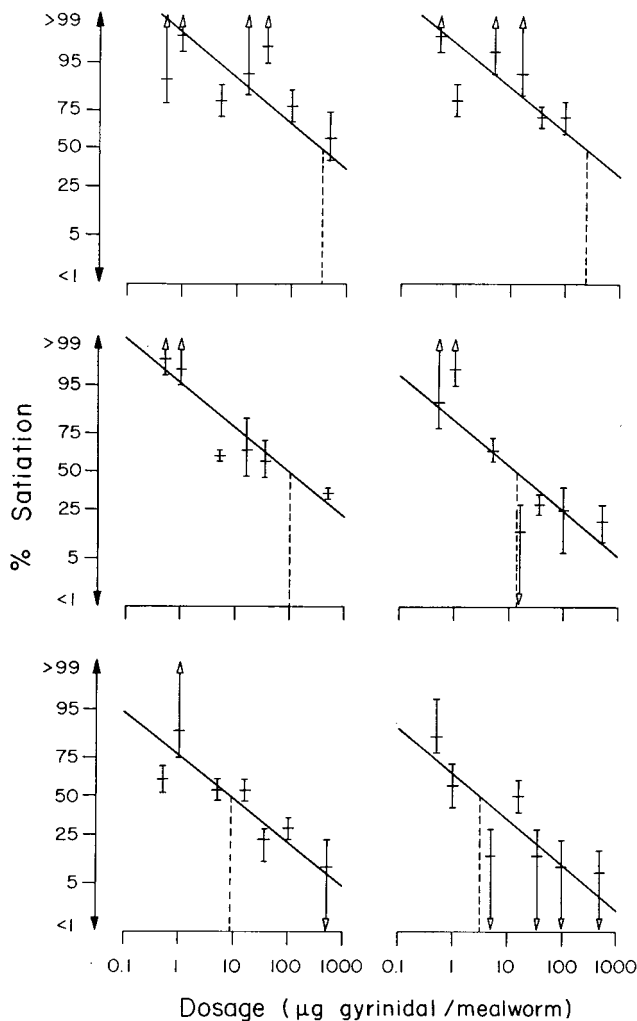


Fig. 6. Oral tolerance of synthetic gyridinal, plotted separately for six bass. Data give the state of satiation of the bass (probit scale) at which given dosages of gyridinal (provided as topical additives to mealworms) were rejected. Dotted lines give dosages predicted to be rejected at 50% satiation. Details in text.

where the first treated item was already rejected in a feeding sequence, it was assumed that last acceptance had occurred at 0% satiation, and the midpoint value was calculated accordingly. By the same token, in cases where all treated items were eaten, it was assumed that rejection occurred at 100% satiation, and the midpoint was calculated on that basis. The gyridinal dosages that predictably would lead the fish to reject treated items when 50% satiated, that is, the oral tolerance limits for gyridinal at that level of satiation, are given by the dotted lines.

It is clear from the plots that the fish varied in their tolerance of gyridinal. Although three of the fish, at 50% satiation, had tolerance limits in the range of 5–10 μg gyridinal, the other three, also at 50% saturation, had tolerance limits of up to over 100 μg . The degree of tolerance did not correlate with size of the fish ($r^2 = 0.05$).

It is also clear that the state of satiation had a profound effect on the acceptability of gyridinal. Dosages accepted by the fish when maximally hungry were higher by about two orders of magnitude than those accepted at near satiation. We recorded acceptance dosages as high as 500 μg gyridinal and rejection dosages as low as 0.5 μg gyridinal.

Duration (Fade-Out Time) of the Beetle's Secretory Emission. As was to be expected, secretory emission proceeded over a longer period in response to ongoing pinching than in response to the single pinch. Signal fade-out time for ongoing stimulation was 97 ± 7 sec (range = 39–154 sec; $n = 20$ beetles). For the single-pinch stimulus, fade-out time was 42 ± 6 sec (range = 12–70 sec; $n = 11$ beetles). There was no difference in the sexes for either value ($P = 0.67$ and 0.72 , for ongoing and single-pinch stimulation, respectively; two-tailed Student's t tests for samples with equal variances).

When the secretion first became visible at the rear of the beetles on discharge, it took on the appearance of a white marginal band around the edge of the pygidium (Fig. 2H), as if it somehow had been trapped at that location. With time, the viscous fluid then drifted away piecemeal with the current (Fig. 2I) until no trace remained around the pygidium. Examination of the *Dineutes* rear with the scanning electron microscope revealed a sieve-like arrangement formed by two sets of interdigitating hairs, one dorsal, the other ventral (Fig. 2L and M), an arrangement that one could envision serving for the temporary retention of secretion. Indeed, the two defensive glands of *Dineutes* open into the space enclosed by these hairs, so one could easily imagine the secretion being trapped in that space and percolating slowly outward through the gaps between the hairs. Observation of the trail of secretion drifting from the beetle's "pygidial sieve" after stimulation had shown that fluid emission from the sieve might not be entirely passive. Output from the sieve was noted to be punctuated by occasional bursts of emission, as if secretion were being forced from within the sieve by mechanical action. There are two palette-like structures (Fig. 2L and N) at the rear of *Dineutes* that might well serve to effect such action. They are present in both male and female and could, by simple retraction-protrusion, press secretion through the sieve. One would expect secretory "puffs" to be emitted in pairs from the sieve by such action, and such was indeed at times seen to be the case (Fig. 2J). The puffs could of course also have been caused by bursts of renewed emission from the glands, but this would not necessarily rule out their additional generation by action of the palettes.

Discussion

The defensive function of gyridinal, certainly vis-à-vis large-mouth black bass, seems established. *Dineutes* itself is largely shunned by this fish, at least in the laboratory, and if taken in the mouth is virtually always spat out before being swallowed. That gyridinal is at least in some measure responsible for the beetle's undesirability is attested to by the compound's proven deterrence, both as part of the beetle's secretion and in pure form, in our bioassays. Existing field data suggest that the results would have been no different with other fish. Whirligig beetles have been found to be virtually absent from the stomach contents of fresh water fish (14, 15).

Not surprising was the finding that the oral tolerance of gyridinal varied over a wide range, as a function of the state of satiation of the bass. Hunger is known to drive many an organism, including fish (16), to feed on what is otherwise rejected.

Of interest are some of the behavioral concomitants of the bass-*Dineutes* interaction, and in particular the fish's apparent standardized tendency to subject gyridinal-bearing food items to oral flushing. We view oral flushing by the bass as a basic activity, possibly used by the fish not merely in its interaction with whirligigs but also as a matter of routine for the preingestive treatment of noxious food items that might be rendered palatable by being flushed in water. The behavior could well be of general occurrence in fish, indicating that these animals took early evolutionary "advantage" of the reality that the fluid they inhabit can be used as a cleansing agent. By the same token, we

view the beetle's secretory delivery system, that is, its propensity to dole out its secretion slowly over time, as having evolved possibly as a consequence of coexistence with fish. What better way to counter oral flushing than to deliver one's deterrent as a trickle? It is interesting that the signal fade-out time for secretory delivery in *Dineutes* is somewhat longer (about 1.5 min, with ongoing stimulation) than the 1 min that the bass spends on average, when half satiated, flushing a food item bearing the gyridal glandular equivalent (100 μg) of a single *Dineutes*. That fade-out time is also longer than the time spent on average by the bass (1.3 min) flushing actual *Dineutes*.

Food ingestion in fish is a complicated process, subject to considerable variation (17–19). It is not unusual for fish to treat different prey items differently and to subject them to various buccal and pharyngeal preingestive processings (19). These behaviors have been studied in considerable detail and could provide a basis for envisioning how oral flushing might have evolved. It would be desirable, however, to establish first whether oral flushing is a general means by which fish deal with chemically noxious prey.

Should oral flushing turn out to be widespread, one might well find aquatic organisms widely to have means for countering the behavior. In organisms that have defensive glands, one might

expect these glands to be adapted for slow delivery of contents, and in those impregnated with noxious chemicals, one would anticipate these chemicals to be built into their bodies in such fashion as not to be readily washed away by flushing.

We have seen oral flushing being executed by other fish in a number of contexts. Swordtails (*Xiphophorus* sp.), for instance, will subject food pellets to oral flushing if the pellets have been treated with some of the defensive pregnanes (1) produced by dytiscid beetles. Similarly, a number of other fish (*Floridichthys carpio*, *Lagodon rhomboides*), when offered pieces of various organs of the sea hare, *Aplysia brasiliensis*, subjected only those organs (body wall, hepatopancreas) to oral flushing that contained some of the defensive halogenated compounds (20, 21) incorporated by the sea hare from its algal diet. The fish swallowed outright, without prior flushing, such *Aplysia* parts (buccal mass) as were free of these compounds (T.E., unpublished data).

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